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Cobalt and Plant Development

INTERACTIONS WITH ETHYLENE IN HYPOCOTYL GROWTH[1]

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ABSTRACT

Co^{2+} promoted elongation of hypocotyl segments of light-grown cucumber (*Cucumis sativus*) seedlings. Time course and dose response data are presented and interactions with IAA, gibberellin, cyclohexanol, and cotyledons described. Segments without cotyledons responded to Co^{2+} only if grown in gas-tight vessels with IAA added. When bases of cotyledons were ringed with an inhibitor of auxin transport, Co^{2+} caused no growth promotion in the hypocotyl. Co^{2+} prevented lateral swelling of hypocotyls treated with supraoptimal IAA. Removal of ethylene from the atmosphere reduced the Co^{2+} response, but Co^{2+} did not counteract the inhibitory effect of increased ethylene levels. These results are consistent with the hypothesis that Co^{2+} promotes hypocotyl elongation by inhibiting ethylene production. The hypothesis was confirmed by a direct demonstration that Co^{2+}, at growth-promoting concentrations, powerfully inhibited ethylene production in the cucumber hypocotyl.

Cobalt salts promote many growth processes, including stem and coleoptile elongation, leaf disc expansion, curvature of slit stems, opening of hypocotyl hooks, and bud development (6, 10, 12, 13, 21). In addition, treatment with Co^{2+} prolongs the critical night period for flowering in *Xanthium* (20). A number of possible mechanisms of Co^{2+} action have been advanced, but most of them (*e.g.* blockage of IAA oxidation) have been adequately refuted by Thimann (21) and Bertsch (2). A more recent hypothesis is that Co^{2+} interferes with both the biosynthesis and the action of ethylene (7). Evidence for an interference with ethylene-induced growth inhibition has been reported by Kang and Ray (7), while workers in three laboratories have observed the inhibition of ethylene biosynthesis by Co^{2+} (8, 11, 16). In the present study of Co^{2+}-induced hypocotyl growth, we have attempted to distinguish between these two forms of interaction of Co^{2+} with ethylene physiology. In addition, we have examined the basis of our earlier observation that green cucumber hypocotyls respond to Co^{2+} only when attached to cotyledon tissue (19).

MATERIALS AND METHODS

Seeds of *Cucumis sativus* L. cv. National Pickling (Burpee Seed Co.) were soaked 2 hr in distilled H_2O and sown in vermiculite. Seedlings grew at 27 C for 5 to 6 days on a cycle of 14L:10D unless otherwise noted. Apical 2-cm segments of the hypocotyls were prepared and incubated in the dark in lots of five in Stender dishes with ground glass covers. Cotyledons and the apical bud remained attached to the hypocotyl segments unless otherwise noted. Each dish contained a filter paper disc and 2 ml of distilled H_2O with or without test additives. The incubation period was 20 hr, except for time course experiments. For the experiments of Figure 4, B and D, dishes were sealed with petroleum jelly to prevent gas exchange.

For pretreatment experiments, hypocotyl segments were incubated for 24 hr in the first test solution, then washed with distilled H_2O, blotted dry, and placed in a second dish with a new test solution for a 2nd day of incubation.

For the experiments summarized in Figure 5, seedlings grew at 27 C for 5 days in absolute darkness. Apical segments of the hypocotyl, including the apical hook but not the cotyledons, were cut under dim green light as described by Purves (17). Segments, initially 5 mm from the basal end to the inside of the hook, were measured again from the basal end to the inside of the hook after 20 hr of growth in the dark.

Ethylene traps were vials containing 1 ml of a mercuric perchlorate solution and a filter paper wick. The solution was prepared by dissolving 5.11 g of $Hg(ClO_4)_2$ in 45 ml of 1.5 M $HClO_4$. $Co(NO_3)_2$ was used as the source of Co^{2+}. Ethrel (2-chloroethylphosphonic acid) was a gift of Amchem Products.

For measurement of ethylene production, lots of 20 1-cm apical hypocotyl segments (from green seedlings), without cotyledons or apical buds, were placed in 4-ml test tubes containing 0.5 ml 1% sucrose, with or without added $Co(NO_3)_2$. The tubes were tightly covered with rubber serum caps. After 3 hr preincubation, 0.05 ml 1 mM IAA was added and the tubes covered again for a 22-hr period of ethylene collection. Throughout the incubation and preincubation periods, the tubes were kept on a roller (12 rpm) to assure adequate aeration and contact with the solution. Ethylene was measured by injecting 10-μl samples of air from the tubes into a Hewlett Packard 5700A gas chromatograph with a flame ionization detector. The column was a Waters Associates "Porapak T," 183 cm × 3.2 mm. Nitrogen at 90 C was the carrier gas. Retention time for ethylene under the conditions employed was 57 sec. Known dilutions of pure ethylene in air were used to construct calibration curves.

RESULTS AND DISCUSSION

Dosage Response Test. Co^{2+} promoted growth over a concentration range of 1 to 500 μM, with a maximum near 0.1 mM (Fig. 1). Concentrations greater than 0.5 mM inhibited growth as compared with water controls.

Time Course of Growth Promotion. The first 5 hr of Co^{2+} treatment yielded no perceptible growth above the controls (Fig. 2A). After this time, when the growth rate of the controls began to decline, the Co^{2+}-treated segments continued to grow at an undiminished rate for the next 5 to 6 hr. By the 12th hr of incubation, almost the full increment of Co^{2+}-induced growth had developed.

Length of Exposure to Co^{2+}. Cobalt treatments of 6 hr or longer within a 24-hr incubation period yielded maximal growth

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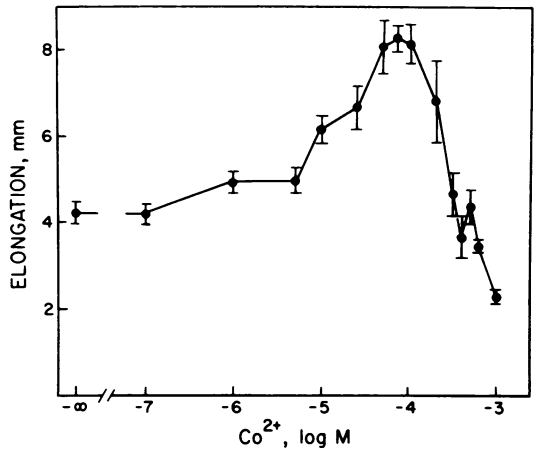

FIG. 1. Elongation of green cucumber hypocotyl segments, with cotyledons attached, as a function of the concentration of $Co(NO_3)_2$.

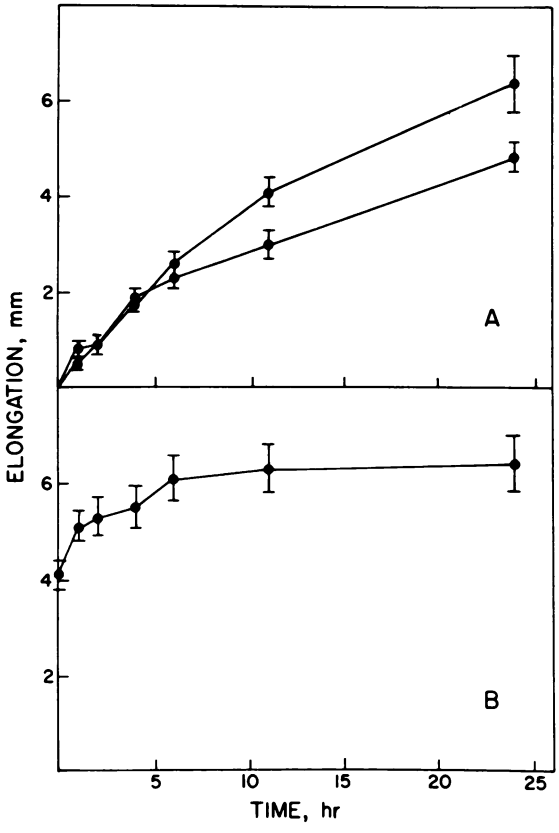

FIG. 2. Time relations in the response of cucumber hypocotyl segments, with cotyledons attached, to 75 μM $Co(NO_3)_2$. A: Time course of growth in water (lower curve) and in Co^{2+} (upper curve); B: Effects of varying durations of exposure to Co^{2+}, measured after 24 hr. Segments were incubated in water after treatment with Co^{2+}.

promotion. Exposures of shorter duration produced responses roughly proportional to the length of treatment (Fig. 2B). Brief exposures to Co^{2+} were not inductive.

Effect of Cotyledons. Unlike etiolated tissue (17), green cucumber hypocotyl segments do not respond to Co^{2+} treatment when the cotyledons are removed (19). Similarly, cucumber hypocotyl segments fail to respond to gibberellins in the absence of the cotyledons (9). However, it appears that the nature of the cotyledon dependence of these two growth regulators is different. In the case of gibberellin, there is a linear dependence of the growth response on the amount of cotyledon tissue left attached

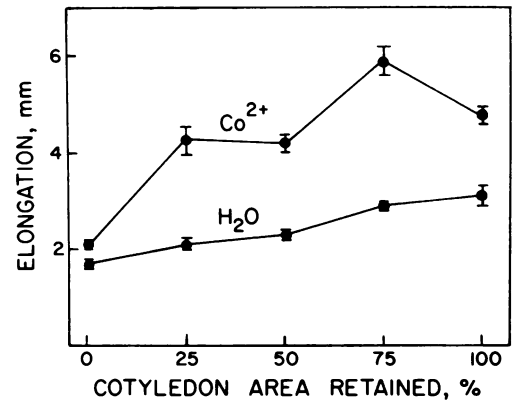

FIG. 3. Effects of cotyledon tissue on cobalt-induced growth of hypocotyl segments. Differing amounts of tissue were excised at the beginning of the incubation period. The concentration of $Co(NO_3)_2$ was 0.1 mM.

(9, and confirmed in the present study). For Co^{2+}-induced growth, the dependence is not linear, and much of the cotyledon tissue can be removed without greatly affecting the Co^{2+} response (Fig. 3). In some tissues, Co^{2+} is synergistic with added sucrose in promoting growth (13, 21). We were unable to cause Co^{2+} responses in segments lacking cotyledons by adding sucrose to the medium, indicating that the cotyledons are not merely serving as a source of photosynthate.

Interactions with Other Growth Promoters. The effect of Co^{2+} in the presence of IAA, gibberellin A_7, and cyclohexanol (18, 19) is shown in Figure 4A. The interaction with auxin and gibberellin is roughly additive, while the interaction with cyclohexanol is negative. No synergisms were observed.

The interaction with IAA was investigated in hypocotyls without cotyledons. When the Stender dish was sealed to contain the atmosphere within the dish, a Co^{2+} response could be elicited in IAA-treated segments (Fig. 4B) even though the cotyledons were detached. In unsealed dishes, IAA did not potentiate a response to Co^{2+}. This suggests that the cotyledons function to supply auxin to the hypocotyl and that conditions which lead to the accumulation of a volatile substance are requisite to a Co^{2+} response.

To test this idea further, hypocotyls with cotyledons still attached were treated with 2,3,5-triiodobenzoic acid,[2] an inhibitor of auxin transport (15). TIBA in lanolin was applied so as to form a ring around each cotyledon at the point of attachment to the hypocotyl. The results (Fig. 4C) indicated a dependence of Co^{2+}-induced growth on the flow of auxin from the cotyledons. Since exogenously supplied auxin functionally replaced the cotyledons only when gas exchange into and out of the incubation vessel was blocked, a logical interpretation of this IAA dependence is an ethylene-Co^{2+} interaction stemming from IAA-induced ethylene production (1, 3, 14).

Effect on Lateral Swelling. Hypocotyls treated with supraoptimal concentrations of IAA responded with a growth pattern characterized by lateral swelling (Table I), a phenomenon caused by IAA-induced ethylene production (3). Pretreatment of the hypocotyl segments with Co^{2+} prior to the addition of IAA prevented the swelling. This, too, is indicative of a cobalt-ethylene interaction.

Effects of Altered Ethylene Levels. The ethylene antagonist theory of Co^{2+} action predicts that Co^{2+} would be without effect if ethylene were removed from the system. When ethylene traps [$Hg(ClO_4)_2$] were added to the sealed Stender dishes, the Co^{2+} response was reduced (Fig. 4D) as predicted. Control growth

[2] Abbreviations: TIBA: 2,3,5-triiodobenzoic acid; CEPA: 2-chloroethylphosphonic acid.

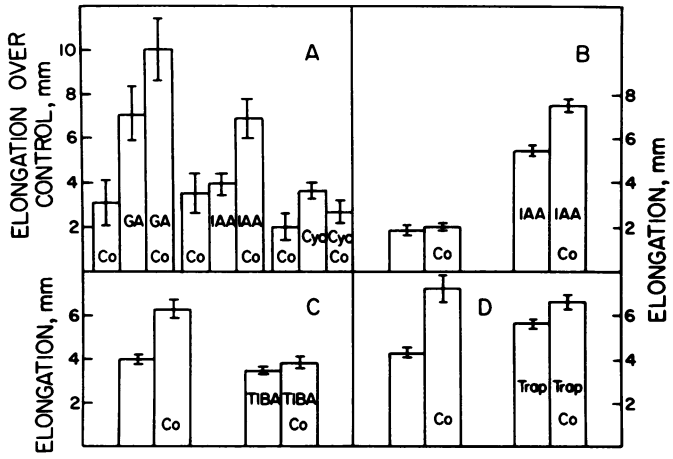

FIG. 4. Interactions of cobalt with various factors on the elongation of hypocotyl segments. A: Interactions of 75 μM $Co(NO_3)_2$ with gibberellin A_7 (0.2 mM), IAA (0.1 mM), and cyclohexanol (0.1%, v/v). Cotyledons present. Bars represent elongation over water controls. B: Ten μM IAA potentiates a response to 0.1 mM $Co(NO_3)_2$ in hypocotyl segments without cotyledons, when test dishes are sealed to prevent gas exchange. C: Triiodobezoic acid (1000 μg/g in lanolin), applied to bases of cotyledons, inhibits the growth response to 0.1 mM $Co(NO_3)_2$. D: Effects of ethylene traps (mercuric perchlorate) on growth of hypocotyl segments (with cotyledons) in water and in 0.1 mM $Co(NO_3)_2$.

Table I. *Effects of Cobalt and Auxin on Lateral Swelling*

Lateral swelling of hypocotyl segments, as measured by mass to length ratio, was determined for various treatment regimes. Incubation media contained distilled H_2O and, where indicated, 0.1 mM $Co(NO_3)_2$ or 1 mM IAA. Cotyledons remained attached to hypocotyl segments during incubation but were removed prior to weighing.

Incubation Medium		Mass/Length
Day 1	Day 2	
		mg/mm
H_2O	H_2O	1.9
Co^{2+}	Co^{2+}	1.9
IAA	IAA	3.0
H_2O	IAA	2.6
Co^{2+}	IAA	2.0

increased as would be expected with a lowered concentration of ethylene.

The growth rate of green hypocotyls without added promoters is so low as to preclude a quantitative study of growth inhibition. Therefore, etiolated hypocotyl segments were used to observe the effects of increased ethylene levels. 2-Chloroethylphosphonic acid markedly inhibited the growth of etiolated hypocotyl segments (Fig. 5), presumably by breakdown of CEPA to ethylene within the tissue (22). Co^{2+}-treated hypocotyls demonstrated a sensitivity to CEPA that paralleled that of the controls, with the Co^{2+}-induced increment relatively constant over a wide range of CEPA concentrations.

Effect of Cobalt on Ethylene Production. Hypocotyl segments treated with sucrose and IAA as described under "Materials and Methods" produced readily measurable quantities of ethylene. In repeated experiments, ethylene production ranged from 20 to 55 nl/g (fresh wt)·hr. When Co^{2+} was added at 0.1 mM or 1 mM, the traces of ethylene detected did not exceed those obtained from control tubes containing no plant material.

DISCUSSION

Reduction of Co^{2+}-stimulated growth through the use of ethylene traps, dependence of the Co^{2+} response on IAA, evidence for the involvement of a volatile compound, and the prevention of IAA-induced lateral swelling by Co^{2+} all support the hypothesis that cobalt reduces ethylene-induced growth inhibition or inhibits ethylene biosynthesis, as proposed by Kang and Ray (7). However, we found no direct evidence that Co^{2+} reduced the sensitivity of tissue to exogenously supplied ethylene. The data of Figure 5, while inconsistent with an interference of Co^{2+} with ethylene action, do conform with a mechanism based on inhibition of ethylene synthesis by Co^{2+}. The mechanism was confirmed by our observation that 1 mM or even 0.1 mM $Co(NO_3)_2$ completely inhibited IAA-induced ethylene production in the cucumber hypocotyl. Inhibition has been reported previously (8, 11, 16), but, to our knowledge, this is the first report of complete inhibition at Co^{2+} concentrations optimal for a physiological process. The proposed mechanism is consistent with all of our data.

In their studies of bean (*Phaseolus vulgaris*) hypocotyl hook opening, Kang and Ray (7) found that added Co^{2+} did overcome the inhibitory effect of added ethylene. This observation contrasts sharply with the data of our Figure 5. It is possible that this discrepancy results from a fundamental difference between bean and cucumber seedlings, or between the straight growth and hook opening responses. However, a unifying hypothesis is also available. It has been shown in several systems that application of ethylene triggers endogenous ethylene synthesis (4, 5). We suggest that the inhibition of hook opening was caused, not by a direct action of the ethylene applied by Kang and Ray (7), but by endogenous ethylene produced in response to that treatment. The reversal by cobalt of the effect of added ethylene could, then, be attributed entirely to an effect of Co^{2+} on ethylene biosynthesis.

Our results (Fig. 4C) also indicate that an important function of cucumber cotyledons is to provide IAA, which in turn causes ethylene production in the hypocotyl. This appears to explain the previously reported (19) requirement for cotyledon tissue in order to obtain a Co^{2+} response in the hypocotyl. The requirement for a sealed container when IAA replaces the cotyledons (Fig. 4B) may reflect an unnatural ventilation of the apical zone when the cotyledons are detached.

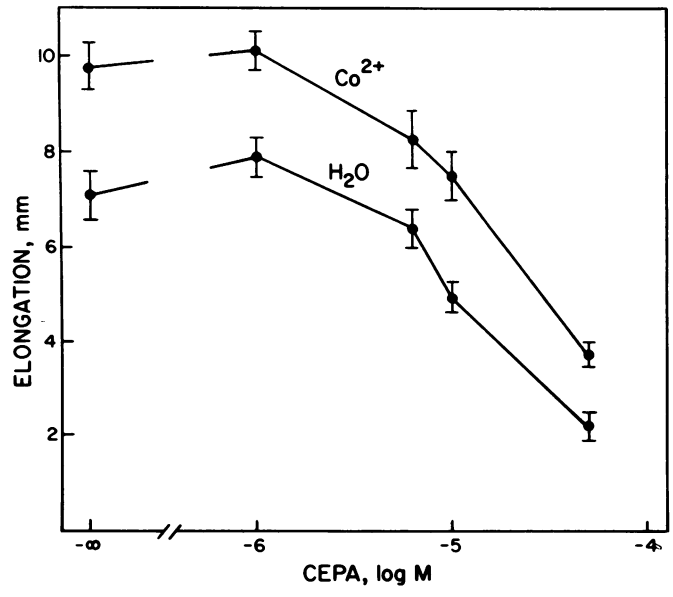

FIG. 5. Responses of etiolated cucumber hypocotyl segments to 2-chloroethylphosphonic acid (an ethylene source) in the presence and absence of 1 mM $Co(NO_3)_2$. Cotyledons were excised. Incubation period: 20 hr.

Delay in the onset of growth elicited by Co^{2+} (Fig. 2A) could be due to penetration problems. Alternatively, ethylene levels may be subinhibitory during the first 5 to 6 hr, since the growth rate of the controls declines only after this point.

Conclusions. (*a*) The promotive effect of Co^{2+} on cucumber hypocotyl elongation (and probably other Co^{2+} effects on plant development) is attributed to the inhibition of ethylene biosynthesis by Co^{2+}. (*b*) The interaction between Co^{2+} and cotyledons is attributed to ethylene produced in response to auxin transported from the cotyledons.

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